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Subcutaneous Vaccination with Attenuated *Salmonella enterica* Serovar Choleraesuis C500 Expressing Recombinant Filamentous Hemagglutinin and Pertactin Antigens Protects Mice against Fatal Infections with both *S. enterica* Serovar Choleraesuis and *Bordetella bronchiseptica*[▽]

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***Salmonella enterica* serovar Choleraesuis strain C500 is a live, attenuated vaccine that has been used in China for over 40 years to prevent piglet paratyphoid. We compared the protective efficacies of subcutaneous (s.c.) and oral vaccination of BALB/c mice with C500 expressing the recombinant filamentous hemagglutinin type I domain and pertactin region 2 domain antigen (rF1P2) of *Bordetella bronchiseptica*. Protective efficacy against both *S. enterica* serovar Choleraesuis infection in an oral fatal challenge model and *B. bronchiseptica* infection in a model of fatal acute pneumonia was evaluated. Both the s.c. and oral vaccines conferred complete protection against fatal infection with the virulent parent *S. enterica* serovar Choleraesuis strain (C78-1). All 20 mice vaccinated s.c. survived intranasal challenge with four times the 50% lethal dose of virulent *B. bronchiseptica* (HH0809) compared with 4 of 20 vector-treated controls and 1 of 18 phosphate-buffered saline-treated controls that survived, but no significant protection against HH0809 was observed in orally vaccinated animals. Both the s.c. and oral vaccines elicited rF1P2-specific serum immunoglobulin G (IgG) and IgA antibodies. However, lung homogenates from s.c. vaccinated animals had detectably high levels of rF1P2-specific IgG and IgA; a much lower level of rF1P2-specific IgG was detected in samples from orally vaccinated mice, and the latter showed no evidence of local IgA. Furthermore, a more abundant and longer persistence of vaccine organisms was observed in the lungs of mice immunized s.c. than in those of mice immunized orally. Our results suggest that s.c. rather than oral vaccination is more efficacious in protecting mice from fatal challenge with *B. bronchiseptica*.**

Bordetella bronchiseptica is an etiological agent of atrophic rhinitis and bronchopneumonia in young pigs. Although the primary disease is important, more significant is the fact that this bacterium predisposes pigs to colonization and disease with other viral and bacterial pathogens (6). *B. bronchiseptica* is also a contributory agent in the porcine respiratory disease complex, a multifactorial disease state that is increasingly problematic for swine producers (2). However, vaccine efficacy is reported to be low, and atrophic rhinitis remains an important disease problem in grower/finisher pigs (1, 34).

Several studies demonstrated that pertactin-specific active or passive immunization against *B. bronchiseptica* protects against mortality and disease in mice and pigs (19, 24, 26). The pertactin protein has two repeated regions, regions 1 and 2; region 2 is identified as being an immunodominant protective epitope (4). The filamentous hemagglutinin (FHA) of *B. pertussis* is defined as being an important attachment factor and protective immunogen (28, 36), with two main immunodominant regions, identified as type I and type II domains (8, 20). In addition, the individual type I domain of FHA induced an immune response that protected BALB/c mice against intranasal (i.n.) infection by the clearance of *Bordetella pertussis* from the lung (18). Because protein structure and immunological analyses suggest that the FHA proteins from *B. pertussis* and *B. bronchiseptica* are similar and have a common set of immunogenic epitopes (21, 27, 30), we hypothesized that a truncation of the FHA of *B. bronchiseptica* that includes the immunodominant type I domain may serve as a protective antigen against porcine bordetellosis.

Over the last decade, the use of recombinant attenuated *Salmonella* vaccine strains for heterologous antigen delivery has increased considerably. A range of strategies has been developed to allow the controlled and stable delivery of antigens and improved immunogenicity where required. The evaluation of different routes of immunization is an important way to modulate immune responses according to clinical requirements. The oral route of antigen delivery is the most common and most frequently explored among the mucosal immunization routes and stimulates both systemic and mucosal immune responses (32, 33). In addition, other immunization routes have been extensively explored in mice, including nasal, rectal, vaginal, and intraperitoneal administration (for examples, see references 7 and 14). However, there have been few previous studies of systemic immunity following subcutaneous (s.c.) vaccination based on this principle.

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TABLE 1. Strains, plasmids, and DNA fragments used in this study

Strain, plasmid, or DNA fragment	Relevant characteristics	Source or reference[a]
Strains		
E. coli		
DH5α	*supE44* Δ*lacU169* (ϕ80*lacZ*Δ*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Takara
BL21(DE3)	F^- *ompT* r_B^- m_B^-; DE3 is a λ derivative carrying *lacI* and T7 RNA polymerase genes under placUV5 control	Takara
χ7213	*thi-1 thr-1 leuB6 fhuA21 lacY1 glnV44* Δ*asdA4 recA1* RP4 2-Tc:Mu[λ*pir*] Km^r	9
χ6097	F^- *ara* Δ(*pro-lac*) *rpsL* Δ*asdA4* Δ[*zhf-2*::Tn*10*] *thi* ϕ80d*lacZ*Δ*M15*	25
S. enterica serovar Choleraesuis		
C500	Live vaccine attenuated from C78-1 by chemical methods; used to prevent piglet paratyphoid in China; serotype 6,7:C:1,5	CIVDC
C78-1	Wild-type, virulent strain	CIVDC
C501	Δ*asd* derivative of C500	This work
C501(pYA-F1P2)	C501 harboring pYA-F1P2	This work
B. bronchiseptica HH0809	Wild-type, virulent strain originally isolated from a pig suffering from atrophic rhinitis	Laboratory stock
Plasmids		
pBluescript SK(+)	Phagemid cloning vector, *ori* ColE1 *oriF1*(+) *bla lacZ*α	Stratagene
pRE112	*oriT oriV* Δ*asd* Cm^r *sacB*; counterselectable suicide plasmid	22
pET28a(+)	IPTG-inducible expression vector; Km^r	Novagen
pET-F1P2	pET28a(+) derivative expressing rF1P2 with an N-terminal His_6 tag; Km^r	This work
pYA3493	Asd^+ vector; pBR322 *ori*; derivative β-lactamase signal sequence-based periplasmic secretion plasmid	17
pYA-F1P2	765-bp DNA encoding the type I region of FHA and R2 repeat domain of pertactin in pYA3493	This work
DNA fragments		
F1	465-bp fragment specifying the important immunodominant type I domain at the carboxy terminus of the FHA gene containing the most reactive epitopes	This work
P2	300-bp fragment specifying the repeated region II domain of the pertactin gene encoding an immunodominant protective epitope	This work

[a] CIVDC, China Institute of Veterinary Drug Control (Beijing, China).

Strain C500 of *Salmonella enterica* serovar Choleraesuis is an avirulent vaccine strain attenuated by chemical methods that is immunogenic and safe and has been used to prevent piglet paratyphoid in China for over 40 years (10, 15, 23). In this work, strain C500 was used as a delivery system for foreign antigens using the Asd^+ balanced-lethal host-vector system (11, 25). We compared the efficacies of oral and s.c. vaccination with the recombinant C500 vaccine strain expressing recombinant filamentous hemagglutinin type I domain and pertactin region 2 domain (rF1P2) antigens of *B. bronchiseptica* in protecting against fatal infections with *B. bronchiseptica* and *S. enterica* serovar Choleraesuis in BALB/c mice. We present data suggesting that s.c. rather than oral vaccination is more efficacious and is sufficient to provide complete protection against both fatal infections in this model.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* and *S. enterica* serovar Choleraesuis cultures were grown at 37°C in Luria-Bertani (LB) broth or on LB agar (3). When required, antibiotics were added to culture media at the following concentrations: ampicillin at 100 μg/ml, kanamycin at 50 μg/ml, and chloramphenicol at 30 μg/ml; DL-α,ε-Diaminopimelic acid (DAP) was added (50 μg/ml) for the growth of Asd^- strains (25). LB agar containing 5% sucrose was used for *sacB* gene-based counterselection in allelic exchange experiments (12). *B. bronchiseptica* HH0809 was grown on Bordet-Gengou agar (Difco, Detroit, MI) supplemented with 15% (vol/vol) defibrinated sheep blood at 37°C.

Expression of rF1P2 in *E. coli*. DNA manipulations were carried out as described previously by Sambrook et al. (31). Transformation of *E. coli* and *Salmonella* was performed by electroporation (Bio-Rad, Hercules, CA). The PCR conditions were as follows: denaturation at 95°C for 30 s, primer annealing at 56°C for 30 s, polymerization at 72°C for 30 s, and a final extension step at 72°C for 10 min. The 465-bp fragment specifying the important immunodominant type I domain (F1) of the FHA gene was PCR amplified from the genome of *B. bronchiseptica* HH0809 using a pair of primers (N-terminal primer 5′-TTTAAGAATTCCTGACTGCCCTGGACAAT-3′ and C-terminal primer 5′-TTTAAGTCGACTCGCAGATCCGCGGCAAA-3′). The N-terminal primer contains an EcoRI site (underlined), and the C-terminal primer contains a SalI site (underlined). The 465-bp amplified fragment, digested with restriction enzymes EcoRI and SalI, was then cloned into the EcoRI and SalI sites of vector pET-28a, resulting in pET-F1. Similarly, the 300-bp fragment specifying the main immunodominant region II (P2) of the pertactin gene was amplified using a pair of primers (N-terminal primer 5′-TAATTGTCGACAACACCATGCTGCTGGTG-3′ and C-terminal primer 5′-TTTAACTGCAGGGCGGACAACTCCCTGCC-3′) and cloned into the SalI and HindIII sites (underlined), respectively, of pET-F1, resulting in pET-F1P2, which harbored a 765-bp fragment of *B. bronchiseptica* HH0809. In-frame cloning of pET-F1P2 was confirmed by nucleotide sequencing using ABI Prism fluorescent Big Dye terminators according to the manufacturer's instructions (PE Biosystems, Norwalk, CT). *E. coli* BL21(DE3) harboring pET-F1P2 was induced by 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at mid-exponential phase and expressed a large amount of His_6-tagged rF1P2 in its cytoplasm. rF1P2 was purified by an affinity purification process with Ni^{2+}-nitrilotriacetic acid-agarose support according to the manu-

facturer's protocol (Qiagen, Valencia, CA). Its immunogenic activity was confirmed by immunoblotting performed as previously described (16), using a serum sample obtained from a pig which had been suffering from atrophic rhinitis and from which *B. bronchiseptica* HH0809 had been isolated.

Preparation of anti-rF1P2 polyclonal antibodies. New Zealand White rabbits (purchased from the Animal Center, Institute of Medicine, Hubei Province, China) were housed in the Experimental Animal Center of Huazhong Agricultural University (Wuhan, China) and were taken care of according to institutional guidelines for the use of animals in research. Rabbits were injected s.c. with 150 μg (0.5 ml) of purified His_6-tagged rF1P2 mixed with an equal volume of complete Freund's adjuvant. The immunization was repeated at 4 and 6 weeks after the first injection. The animals were bled 2 weeks after the third injection, and serum was collected by centrifugation at 1,700 × *g* for 10 min and stored at −80°C until use.

Construction of an *S. enterica* serovar Choleraesuis C500 vaccine strain with an *asd* deletion. The 2,112-bp upstream fragment of the *asd* gene was amplified from the genomic DNA of *S. enterica* serovar Choleraesuis strain C500 using a pair of primers (N-terminal primer 5′-TTTCTAGACGCTTTGAGCACGACT AA-3′ and C-terminal primer 5′-TTGGATCCTGCGTTAGGAAGGGAAT C-3′ [the XbaI and BamHI sites, respectively, are underlined]) (GenBank accession no. AE008863) by PCR performed as described above, with the exception of polymerization at 72°C for 2.5 min. The PCR product was cloned into the XbaI and BamHI sites of the pBluescript II SK(+) vector, resulting in pSK-asd^{up}. The 2,069-bp downstream fragment of the *asd* gene was then PCR amplified using a pair of primers (5′-TTGGATCCAGGGTAGCTTAATCCCA C-3′ and 5′-TTGGTACCACCGAGCGTTCATTGTCA-3′) and cloned into the BamHI and KpnI sites (underlined), respectively, of pSK-asd^{up} to obtain pSKΔasd, which resulted in a 1,408-bp deletion including the *asd* gene fragment. The 4,181-bp fragment, including the upstream and downstream fragments of the *asd* gene, from XbaI- and KpnI-digested plasmid pSKΔasd was ligated into plasmid pRE112 to yield suicide plasmid pREΔasd. Transfer of recombinant suicide plasmids into *S. enterica* serovar Choleraesuis C500 was accomplished by conjugation using *E. coli* χ7213 (Asd$^-$) (9) as the plasmid donor. Strains containing single-crossover plasmid insertions (C500asd::pREΔasd) were isolated on plates containing chloramphenicol. A loss of the suicide vector after the second recombination between homologous regions (i.e., allelic exchange) was selected for by using the *sacB*-based sucrose sensitivity counterselection system (12). The presence of the 1,408-bp *asd* deletion in *S. enterica* serovar Choleraesuis C500 was confirmed by the inability of the strain to grow on medium without DAP (25) and by PCR using a flanking *asd* primer set (5′-TTGCTTTCCAACTGCTGAG C-3′ and 5′-TCCTATCTGCGTCGTCCTAC-3′).

Characterization of phenotype. The 765-bp fragment was purified from EcoRI- and HindIII-digested plasmid pET-F1P2 and cloned into the EcoRI and HindIII sites of pYA3493, resulting in pYA-F1P2. In-frame cloning of pYA-F1P2 was confirmed by nucleotide sequencing. pYA3493 (vector control) and pYA-F1P2 (encoding rF1P2) were electroporated into the Δ*asd* C500 strain (named C501), resulting in recombinant *S. enterica* serovar Choleraesuis strain C501(pYA-F1P2) and vector control strain C501(pYA3493).

The growth rates of the strains in LB broth were tested, and carbohydrate fermentation or utilization assays were conducted using commercial biochemistry tubes according to the manufacturer's protocol (Tianhe, Hangzhou, China). The presence of group O and group H antigens was confirmed by slide agglutination with antisera supplied by the China Institute of Veterinary Drug Control (Beijing, China). The expression of the rF1P2 antigen in the cytoplasm and culture supernatant of C501 was checked by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and immunoblot analyses were performed with anti-rF1P2 rabbit polyclonal antibody as previously described (16). Band intensities were analyzed densitometrically using the Bio-Rad Quantity One program (Bio-Rad Laboratories, Hercules, CA).

Immunization and sampling. Four- to five-week-old female BALB/c mice (purchased from the Animal Center of Hubei Institute of Medicine, Wuhan, China) were housed in filter-top cages in an air-conditioned animal facility in the Experimental Animal Center of Huazhong Agricultural University (Wuhan, China) and were taken care of according to institutional guidelines for the use of animals in research. Immunization doses were chosen according to previously published data (15, 23). For oral vaccination, mice were deprived of food and water for 4 h and were then given 50 μl of 10% sodium bicarbonate solution by orogastric gavage with a 12-gauge ball-tipped gavage needle. Ten minutes later, mice were fed 200 μl of either phosphate-buffered saline (PBS) or *Salmonella* vector C501(pYA3493), recombinant vaccine C501(pYA-F1P2), or the parent C500 vaccine strain (2.1×10^{10} CFU in PBS) by the same method, and food and water were returned 30 min after inoculation. For s.c. vaccination, mice were inoculated s.c. on the dorsum with either 200 μl PBS, *Salmonella* vector C501(pYA3493), recombinant vaccine C501(pYA-F1P2), or the parent C500 vaccine strain (2.1×10^8 CFU in 200 μl PBS). After 14 days, all animals were boosted with the same dose of the appropriate vaccine.

Serum, lung, and gut mucus samples were collected on days 0, 28, and 56. Five animals from all groups before vaccination and five animals from each group after vaccination were anesthetized with an intraperitoneal injection containing xylazine (0.5 mg) and ketamine (2.5 mg) and then bled thoroughly by removing the eyeball. Additional blood samples were collected from the retro-orbital sinuses of mice in groups of five for monitoring the kinetics of serum antibody responses on days 14 and 42. Serum was collected by centrifugation at 1,700 × *g* for 10 min and then stored at −80°C until use. To collect gut mucus, mice were sacrificed, and the gut was removed between just distal to the stomach and just proximal to the anus. The mucus was scraped from the luminal surface and suspended in 1 ml PBS. The lung was removed aseptically and homogenized in 2 ml PBS. After centrifugation at 12,000 × *g* for 5 min at 4°C, supernatant fluids from gut mucus and lung homogenates were collected, and samples were analyzed immediately using an indirect enzyme-linked immunosorbent assay (ELISA).

For in vivo localization of bacteria, groups of four mice were sacrificed on days 2, 8, and 14 after the initial immunization. Lungs, spleens, and Peyer's patches were removed aseptically. The tissues were homogenized in sterile PBS and plated onto MacConkey agar (Tianhe, Hangzhou, China) plates with or without 1% maltose to examine the distribution and persistence of the recombinant vaccine.

Intranasal infection with *B. bronchiseptica*. A highly virulent strain of *B. bronchiseptica*, HH0809, originally isolated by our laboratory from a pig suffering from atrophic rhinitis, was used for the challenge of mice on day 30 after the initial immunization. HH0809 cells were grown on Bordet-Gengou agar for 48 h as described above. Bacteria were resuspended and diluted in 1% Casamino Acids and then serially diluted to provide challenge inoculum dilutions. For respiratory infection, 20 μl of the bacterial suspension containing approximately four times the 50% lethal dose (LD_{50}) of virulent strain HH0809 (5.2×10^6 CFU) was deposited into each nostril of mice that had been anesthetized with an intraperitoneal injection containing xylazine (0.25 mg) and ketamine (1.25 mg). Morbidity and mortality were observed for 30 days after the challenge.

ELISA for *Salmonella* and rF1P2. An ELISA was used to assay antibodies to whole *Salmonella* cells or to rF1P2 in samples of serum, lung homogenate, and intestinal mucus from individual mice. Each sample well of polystyrene 96-well flat-bottomed microtiter plates (Kangjia Ltd., China) was coated with 100 ng of purified rF1P2 diluted in 100 μl 0.1 M carbonate buffer (pH 9.6). For anti-*Salmonella* antibody titration, *S. enterica* serovar Choleraesuis C500 cells were grown overnight, harvested by centrifugation, and resuspended in PBS at 3×10^{11} CFU/ml. Bacteria were heat killed for 10 min at 80°C and stored at −80°C. Each sample well was coated with 100 μl of this suspension diluted 100-fold in carbonate buffer. The coated plates were incubated at 37°C for 1 h, followed by an overnight incubation at 4°C. Free binding sites were blocked with a blocking buffer (PBS, 0.1% Tween 20, and 5% skim milk). Samples of serum, lung homogenate, or gut mucus were added to each well and incubated at 37°C for 30 min. After three washes, plates were treated with biotinylated goat anti-mouse immunoglobulin G (IgG) (Southern Biotechnology Inc., Birmingham, AL) for sera and lung homogenates, or IgA for all samples, at 37°C for 30 min, followed by five washes. Substrate solution containing TMB (3,3′,5,5′-tetramethylbenzidine) and H_2O_2 (50 μl) was then added to each well and incubated at room temperature in the dark for approximately 10 min; the catalytic reaction was stopped by adding 50 μl 1% SDS. The optical density was read at 630 nm using an ELISA reader.

Statistics. All analyses were performed by use of SAS system 8.1 software. *Salmonella*- and rF1P2-specific IgG and IgA titers and numbers of vaccine organisms recovered in murine tissues in $\log_{10}$ units were compared by a Student's *t* test. For survival studies, data were analyzed by Fisher's exact test. In these two tests, a *P* value of <0.05 was considered to be significant.

RESULTS

Characterization of recombinant *S. enterica* serovar Choleraesuis C500 vaccine expressing rF1P2. *S. enterica* serovar Choleraesuis C500 *asd* deletion mutant C501 lost the ability to synthesize DAP and was unable to grow on medium without DAP. Its ability was restored when C501 harbored plasmid pYA3493 or pYA-F1P2, resulting in C501(pYA3493) and C501(pYA-F1P2). The mean generation times of recombinant

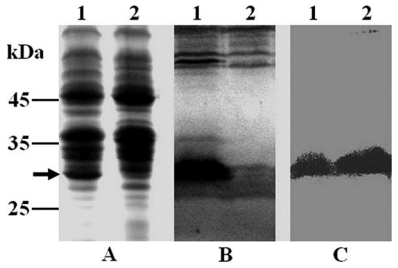

FIG. 1. Expression of rF1P2 in *S. enterica* serovar Choleraesuis C501. Vaccine strain C501(pYA-F1P2) and vector control strain C501(pYA3493) were cultured in LB broth at 37°C. Total cells (equivalent to 1.2×10^9 cells) and concentrated culture supernatants (equivalent to 750 μl of culture supernatant at an optical density at 600 nm of 0.8) were subjected to SDS-polyacrylamide gel electrophoresis analysis, and rF1P2 was detected by immunoblotting with anti-rF1P2 rabbit polyclonal antibody. (A) Coomassie brilliant blue-stained gel of total cell extracts of C501(pYA-F1P2) (lane 1) and C501(pYA3493) (lane 2). (B) Immunoblot of the duplicated gel (A) with anti-rF1P2 rabbit polyclonal antibody. (C) Immunoblot of total cell extract (lane 1) and concentrated supernatant (750 μl) (lane 2) of C501(pYA-F1P2). Molecular markers are indicated to the left. rF1P2 proteins are indicated by an arrow.

S. enterica serovar Choleraesuis C501(pYA-F1P2), the vector control C501(pYA3493), and the parent avirulent C500 vaccine strain in Luria broth were 30.7, 28.1, and 27.9 min, respectively. The fermentation patterns of the various strains on different carbohydrates, and the levels of production of H_2S, were similar. The O and H antigens of C501(pYA3493) and C501(pYA-F1P2) were 6,7:C:1,5, identical to the parent strain C500. Recombinant strain C501(pYA-F1P2) expressed the rF1P2 protein at an approximate molecular mass of 30 kDa, consistent with the calculated size of rF1P2 (Fig. 1). Analysis of Coomassie blue-stained SDS-polyacrylamide gels showed that the amount of the rF1P2 protein accounted for up to approximately 1.7% of the total C501(pYA-F1P2) protein; approximately 71.4% of rF1P2 was located in the cell lysates, and 28.6% was located in the culture supernatants. To examine the stability of plasmids pYA3493 and pYA-F1P2 in C501 in vitro, C501 cells containing pYA3493 and pYA-F1P2 were cultured with a daily passage of 1:1,000 dilutions for five consecutive days in LB broth containing DAP. Cells obtained from the last-day culture expressed amounts of the 30-kDa rF1P2 that were similar to those from the first day (data not shown), suggesting the stable expression of rF1P2 without rearrangements.

Immunogenicity to *S. enterica* serovar Choleraesuis. All immunized mice survived, and no signs of disease in the immunized mice were observed during the entire experimental period. The recombinant C501(pYA-F1P2) vaccine elicited IgA and IgG antibody responses with levels similar to those induced by C500 by either the oral or the s.c. inoculation route (data not shown). When the anti-*Salmonella* IgG and IgA titers induced by the s.c. and oral routes were compared, s.c. immunization with C501(pYA-F1P2) elicited lower levels of IgA (titer, 48) in the gut mucus but a significantly higher serum IgG antibody level (titer, 3,584 [ninefold] [$P < 0.01$]) than oral immunization (IgA and IgG titers, 144 and 384) 4 weeks following the initial immunization. Thirty days after the initial immunization, groups of mice were challenged with different amounts (from 10 to 10^4 times the oral LD_{50}) of wild-type virulent parent strain C78-1 by the oral route. Deaths were recorded for 30 days, and the LD_{50} after immunization was calculated by a method described previously by Reed and Muench (29). No major differences between the protection afforded by C501(pYA-F1P2) and that afforded by C500 were found, regardless of the route of vaccination. Both the s.c. and oral immunization routes completely protected mice against a subsequent oral challenge with 100 times the LD_{50} of C78-1, whereas there were no survivors in a group of 10 PBS controls. The LD_{50} after s.c. immunization with C501(pYA-F1P2) was $\log_{10}$ 8.1 (equal to that obtained when animals were immunized with C500), and the LD_{50}s were $\log_{10}$ 7.6 and $\log_{10}$ 7.4 after oral immunization with C501(pYA-F1P2) and C500, respectively. These results indicate that s.c. immunization with C501(pYA-F1P2) or C500 is avirulent for mice and can provide complete protection from *S. enterica* serovar Choleraesuis infection.

Antibody responses to rF1P2 in sera. The IgG and IgA responses in sera were assessed following two doses of vaccine (Fig. 2). The kinetics of the rF1P2-specific serum IgG and IgA antibody responses of vaccine-inoculated mice were monitored and compared with those of PBS- and vector-treated mice (data not shown). Primary immunization with C501(pYA-F1P2) given s.c. induced significant levels of serum anti-rF1P2 IgG and IgA within 2 weeks. Serum samples taken 2 weeks after booster immunization showed a further increase in the levels of rF1P2-specific IgG and IgA; the highest total IgG and IgA titers were observed 4 and 2 weeks after booster immunization. A similar trend was observed in orally vaccinated mice, except that the highest IgG and IgA titers were observed 6 and 4 weeks after booster immunization. It should be noted that s.c. immunization with C501(pYA-F1P2) elicited lower levels of serum IgA (Fig. 2B) but induced significantly higher ($P < 0.01$) serum IgG antibody levels than oral immunization with titers of 3,584 and 14,336 on days 28 and 56 (Fig. 2A).

***B. bronchiseptica* wild-type challenge.** In the survival study, mice inoculated with vector and PBS, regardless of the route of immunization, died between days 2 and 7, with the peak of mortality on day 4, after challenge with highly virulent *B. bronchiseptica* HH0809. Complete protection (20/20) over a 30-day observation period was seen in mice that had received the recombinant vaccine by the s.c. route; the survival rates were much lower in mice that received either PBS (1/18) or vector (4/20) alone (Table 2). In addition, no obvious signs of disease were observed in the mice immunized s.c. with C501(pYA-F1P2) during the entire experimental period. However, i.n. challenge of orally vaccinated mice with HH0809 (5.2×10^6 CFU) failed to produce a significant difference in survival rates between vaccine-treated mice and PBS- and vector-treated controls (Table 2), even though these mice showed a significant increase in survival as a function of time postinfection compared with the vector- or PBS-treated controls (data not shown). Altogether, these results suggest that vaccine strain C501(pYA-F1P2) provides complete protection against challenge with virulent wild-type *B. bronchiseptica* HH0809 and is more efficacious when delivered by the s.c. route.

Antibody responses to rF1P2 in lung. Having produced evidence indicating that s.c. rather than oral immunization provides complete protection in the i.n. challenge model, we

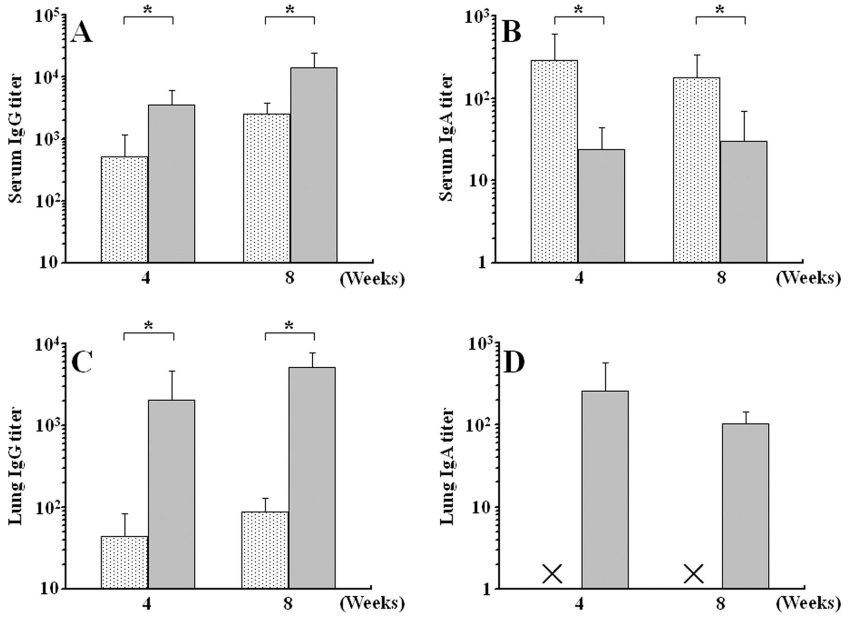

FIG. 2. ELISA of immune responses in mice vaccinated with recombinant *Salmonella* C501(pYA-F1P2) vaccine by the s.c. or oral route. (A) Serum anti-rF1P2 IgG titers. (B) Serum anti-rF1P2 IgA titers. (C) Lung anti-rF1P2 IgG titers. (D) Lung anti-rF1P2 IgA titers. Mice were inoculated with *S. enterica* serovar Choleraesuis vaccine strain C501(pYA-F1P2) or vector strain C501(pYA3493) on days 0 and 14. Samples from five mice were collected at 4 and 8 weeks after primary immunization given s.c. (filled bars) or orally (light patterned bars). Individual samples of mouse sera and lung homogenates were tested for total IgG and IgA antibodies against rF1P2 by ELISA. The titers represent the maximum end-point dilutions from the sample yielding an optical density at 630 nm two times that of undiluted sera from the vector-treated mice. Under these experimental conditions, samples from the vector-treated mice always gave an optical density at 630 nm of <0.1 from the first dilution. "×" represents no detectable antibody levels of titers of ≥10. Mean values for each group were compared using a Student's *t* test. Error bars indicate standard deviations. *, $P < 0.01$ between the s.c. and orally inoculated groups.

wanted to determine whether a difference in the antibody profiles existed at the site of infection after either vaccination. To accomplish this, lung homogenates were obtained from mice after s.c. and oral inoculation. In mice that received the s.c. vaccine, high levels of total IgG and IgA were observed in lung homogenates, whereas only low-level reactivity for total IgG was seen in orally vaccinated animals, with no evidence of rF1P2-specific IgA (titer, <10) (Fig. 2C and D). Interestingly, the IgA level in lung homogenates was significantly higher ($P < 0.01$) than that in sera 4 and 8 weeks following s.c. immunization (Fig. 2B and D). These results suggest that local lymphoid tissues in lung may be a source of protective antibodies, in addition to transudation from the circulation. The lack of IgA in lung homogenates from orally vaccinated mice was unexpected, as this antibody was observed in serum samples from these animals. These results suggest that not only IgG but also IgA in lung tissue may play an important role in mediating protection in s.c. vaccinated mice.

TABLE 2. Effectiveness of s.c. or oral immunization with recombinant *S. enterica* serovar Choleraesuis vaccine strain C501(pYA-F1P2) in protecting BALB/c mice against i.n. challenge with wild-type *B. bronchiseptica* HH0809

Strain (genotype) or control[a]	Inoculation route	Immunization dose (CFU)	Challenge dose (CFU)[b]	No. of survivors/ total no. of mice
C501(pYA3493)	s.c.	2.1×10^8	5.2×10^6	4/20
C501(pYA-F1P2)	s.c.	2.1×10^8	5.2×10^6	20/20[c]
PBS	s.c.	200[d]	5.2×10^6	1/18
C501(pYA3493)	Oral	2.1×10^{10}	5.2×10^6	4/18
C501(pYA-F1P2)	Oral	2.1×10^{10}	5.2×10^6	4/20
PBS	Oral	200[d]	5.2×10^6	3/18

[a] Mice were s.c. or orally immunized twice at 2-week intervals with the indicated vaccine strains or with PBS and challenged 30 days after the primary immunization with wild-type *B. bronchiseptica* HH0809. Morbidity and mortality observations were recorded daily for 30 days postchallenge.

[b] A total of 5.2×10^6 CFU represents about four times the LD_{50} of HH0809 in nonimmunized BALB/c mice.

[c] $P < 0.0001$ by Fisher's exact test for vector versus vaccine, PBS versus vaccine, and s.c. versus oral vaccination.

[d] Value in microliters.

In vivo distribution of vaccine organisms. The presence of live C501(pYA-F1P2) vaccine organisms was then observed in lungs, spleen, and Peyer's patches following both s.c. and oral delivery (Table 3). In these two groups, similar numbers of organisms were isolated from the Peyer's patches of mice after inoculation. In contrast, the numbers of vaccine colonies isolated from spleens of mice inoculated s.c. were significantly higher than in those of mice inoculated orally on days 2, 8, and 14 following immunization. Most importantly, much larger numbers of vaccine colonies were isolated from the lungs of s.c. immunized mice on day 2, with a significant increase on day 8 and then a rapid decline on day 14. However, only smaller numbers of vaccine colonies were detected on day 2, and no organisms were detected in lungs of mice immunized orally on days 8 and 14, suggesting the temporary persistence of vaccine organisms in murine lungs. Interestingly, very similar numbers of organisms were isolated from the lungs, spleen, and Peyer's

TABLE 3. Persistence of recombinant *S. enterica* serovar Choleraesuis vaccine strain C501(pYA-F1P2) in deep organs of BALB/c mice[a]

Route	Log_{10} CFU recovered from:								
	Lungs on day:			Spleen on day:			Peyer's patches on day:		
	2	8	14	2	8	14	2	8	14
s.c.	2.7*	3.4	1.4	2.6*	3.2*	1.7	2.6	3.3	2.5
Oral	1.6	ND	ND	1.9	<1[b]	ND	2.8	3.5	2.3

[a] Mice were inoculated orally or s.c. with a single dose of 2.1×10^{10} or 2.1×10^{8} CFU C501(pYA-F1P2) organisms. Data are averages of values for four mice, and ND represents no detectable organisms. *, $P < 0.01$ by Student's t test between the s.c. and orally inoculated groups.
[b] One mouse had low counts of vaccine organisms, and the other three mice had cleared all organisms.

patches in s.c. immunized mice at day 2 or 8 after immunization (Table 3). Parent strain C500 localized in the same tissues, and no statistical differences were observed in the counts of the live bacteria alone compared to bacteria carrying plasmid pYA-F1P2 (data not shown).

DISCUSSION

S. enterica serovar Choleraesuis strain C500 is an avirulent vaccine strain attenuated by chemical methods, which is highly immunogenic and safe and has been used widely to prevent piglet paratyphoid in China for over 40 years (10, 15, 23). In this study, all mice immunized s.c. with C500 or C501(pYA-F1P2) survived, and no signs of disease were observed in the immunized mice during the entire experimental period. Either s.c. or oral vaccination in BALB/c mice induced immune responses to both *Salmonella* and rF1P2 and provided effective protection against fatal challenge with a virulent *S. enterica* serovar Choleraesuis strain, C78-1. These results indicate that s.c. vaccination with C500 or C500 with an Asd^+ plasmid is avirulent for mice. Furthermore, rF1P2-specific immunity did not interfere with immunity against *Salmonella* itself. Previous works on the protective efficacy of FHA and pertactin revealed that a single antigen or an immunodominant protective domain alone may provide sufficient protective efficacy against *Bordetella* challenge if effective immunity can be induced via vaccination (18, 19, 24, 28). In this study, the secretion of the rF1P2 antigen expressed in *Salmonella* was confirmed, which may augment immune responses by facilitating the adequate exposure of rF1P2 antigen to antigen-presenting cells for processing (17).

Although routes of delivery of antigens expressed in recombinant *Salmonella* strains have been extensively explored in mice (32, 33), there were few previous studies demonstrating that s.c. vaccination produced effective immune responses and protection based on this principle. In this study, the results from immunization experiments demonstrated that s.c., but not oral, vaccination with this strain provided complete protection against i.n. challenge with *B. bronchiseptica*, which is supported by much higher anti-rF1P2 IgG and IgA levels detected in lungs of mice following s.c. but not oral inoculation. These findings suggest that protection against i.n. infection correlates with the local systemic responses in murine lungs elicited by s.c. vaccination. These findings also indicate that the degree of activation of gut-associated lymphoid tissue by oral vaccination is insufficient for antibody-secreting B cells to localize to the respiratory lymphoid tissue based on this principle, even though mice immunized orally showed an increase in survival as a function of time postinfection (data not shown). The sources of anti-rF1P2 IgG and IgA antibodies in the respiratory tract after s.c. immunization have not yet been directly determined; however, we suspect that local lymphoid tissues may be a source of the protective antibodies rather than transudation from the circulation alone. This interpretation is based on the findings that oral vaccine promoted a potential total anti-rF1P2 IgA response in serum but not in lung and that s.c. immunization induced significantly higher anti-rF1P2 IgA antibody levels in lung homogenates than in sera ($P < 0.01$).

We then performed kinetic bacterial distribution assays of murine tissues after inoculation. The current finding that the vaccine organisms persisted more abundantly and longer in the lungs and spleen of s.c. inoculated mice suggests that, for some reason, the vaccine organisms might reach these murine tissues more effectively following s.c. inoculation than following oral inoculation. This persistence of vaccine organisms in the lungs is in agreement with the greater immunogenicity of the s.c. immunization. In contrast, orally inoculated mice had only a short persistence of vaccine organisms, which might be not sufficient to stimulate antibody responses in the lungs, and this likely accounted partially for the poor antibody responses against the heterologous antigen in this local tissue. The stronger persistence of vaccine organisms in the Peyer's patches may result from the environment, which is less hostile than that in either the lungs or the spleen of mice based on tissue specificity (13). Simultaneously, large numbers of organisms were isolated from the lungs, spleen, and Peyer's patches of mice following s.c. inoculation, suggesting that the s.c. inoculum may establish a reservoir in the lymph nodes, where *Salmonella* readily spreads throughout the body via the lymph stream and becomes systemic (5, 35), finally reaching the lungs and other tissues for the generation of systemic and local immune responses.

In conclusion, we have shown that s.c. vaccination with recombinant attenuated *Salmonella* vaccine strain C500 is a more suitable immunization route than oral immunization for the induction of protective immune responses against fatal infections with both *S. enterica* serovar Choleraesuis and *B. bronchiseptica* in this model. It is likely that this *Salmonella* expression and delivery system could be easily adapted to develop multivalent recombinant *Salmonella* vaccines against other infectious agents. Further work is needed to determine the potential of the vaccine in pigs before comprehensive evaluation and practical application are done. In addition, it would be interesting to compare the protective efficacy of s.c. vaccination

with recombinant *Salmonella* vaccine strain C500 to that of i.n. vaccination.

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